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D. E. SALMON, D. V. M., Chief of Bureau.

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STUDIES UPON THE

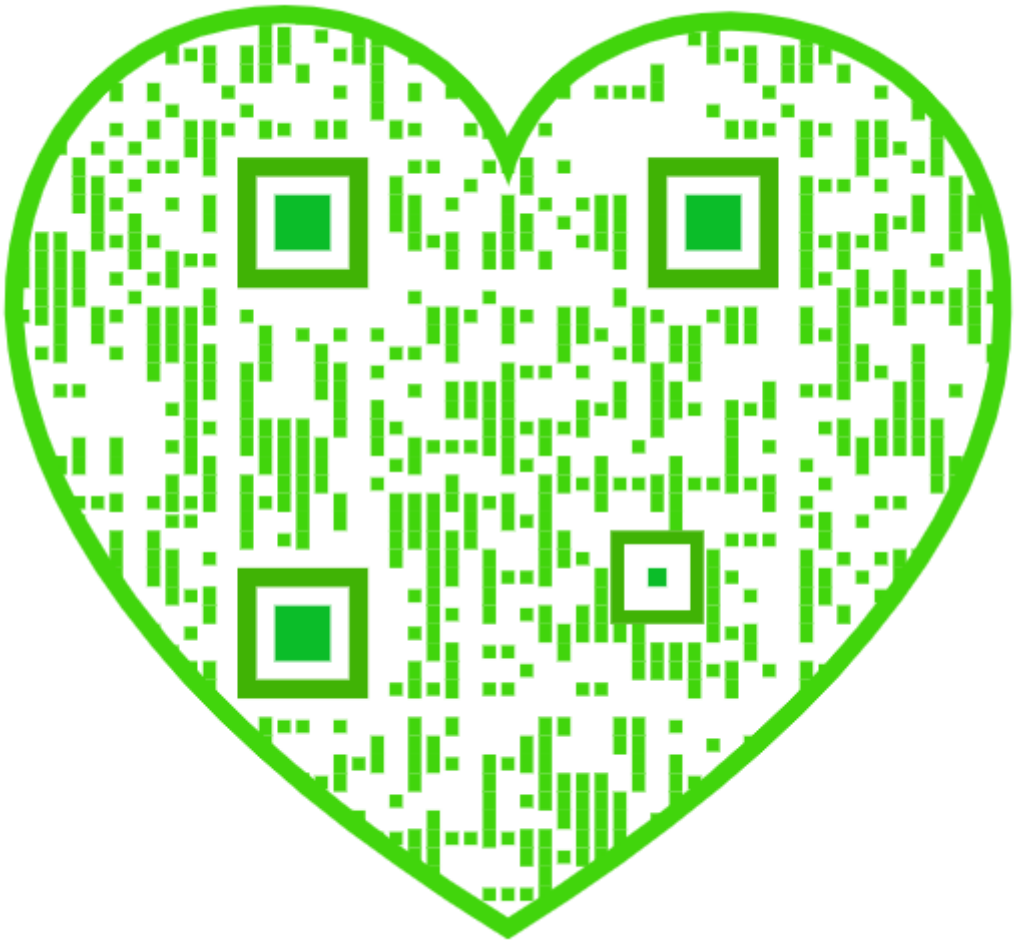
KEEPING QUALITY OF BUTTER.

I.—CANNED BUTTER.

BY

LORE A. ROGERS,
Expert in Dairy Bacteriology, Bureau of Animal Industry.

WASHINGTON:
GOVERNMENT PRINTING OFFICE.
1904.



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LETTER OF TRANSMITTAL.

U. S. Department of Agriculture,
Bureau of Animal Industry,
Washington, D. C., January 12, 1904.

Sir: I have the honor to transmit herewith a manuscript entitled "Studies upon the keeping quality of butter," by Mr. Lore A. Rogers, expert in dairy bacteriology. This paper deals with the particular feature of changes occurring in canned butter, but a second paper will soon be submitted on the subject of packed butter. I recommend that the manuscript herewith transmitted be published as a bulletin in the series of this Bureau.

Respectfully,

D. E. Salmon, *Chief of Bureau.*

Hon. James Wilson, *Secretary.*

OCT 20 1906
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THE KEEPING QUALITY OF BUTTER.

I.—CANNED BUTTER.

By Lore A. Rogers,
Expert in Dairy Bacteriology, Bureau of Animal Industry.

In taking up the study of abnormal changes in butter, the possible decomposition of three groups of chemical compounds must be considered.

The sugar, which has already been partly fermented during the ripening of the cream, breaks up most readily into lactic and similar acids, which, without giving the butter any unpleasant taste, protect it from other fermentations by preventing the growth of bacteria less resistant to these acids than the group of bacteria by which they were produced.

In addition to the sugar there is in the butter a small amount of casein and, in solution in the water, albumen, together with small quantities of the decomposition products of these two proteids. The curd is made up by the insoluble casein, and all are usually grouped together in the chemical analyses under the heading "casein," or "nitrogenous constituents." Under ordinary circumstances this group is subject to decompositions of a widely varying nature. In butter, however, the growth of the putrefactive bacteria, through whose agency these changes are usually brought about, is checked by the presence of the lactic acid which has been produced by the fermentation of the sugar. Under certain circumstances, as for instance, an imperfect ripening of the cream, the nitrogenous constituents may undergo an active decomposition, with a consequent spoiling of the product. This is probably a more unusual trouble than is generally believed and is easily distinguished from the more common troubles caused by changes in the fat.

The third substance, the fat, which makes up between 80 and 90 per cent of the butter, is not a single compound, but a mixture of a considerable number of closely related but distinct fats. These fats, or glycerides, which are formed by the chemical union of a fatty acid with glycerine, when mixed in the proportion found in butter, have an agreeable taste and smell. These glycerides are

somewhat unstable, and some of them are easily decomposed. The fatty acids, while classed with the weakest of the organic acids, are characterized by their peculiar pungent odor and taste. When, through any abnormal fermentation, one or more of these glycerides is broken down, the fatty acid is set free, and, even though it may be present in very small quantities, it gives the butter a peculiar disagreeable flavor varying probably in its nature and intensity with the kind and amount of the acid liberated.

The "off" flavors found in butter present all gradations between the indistinct "not fresh" flavor and the great variety of disagreeable odors and tastes which are sometimes indiscriminately designated by the term "rancidity." As in all questions relating to flavors, it is very difficult to find two persons agreeing on just what constitutes "rancidity." Some investigators have understood rancidity to mean any change from the normal, while others use the term to designate only the "off" flavors caused by a certain amount of free fatty acid. A great variety of opinion may be found on this subject. In this paper the term "rancidity" is not used, partly because of this difference of opinion and partly because few, if any, of the butters mentioned here had reached a state of decomposition that most investigators and dairymen would consider as rancidity.

The question of the decomposition of butter fat has received more or less attention, but until recently has been surrounded with considerable obscurity. The decomposition has been ascribed by different investigators to various causes, particularly to oxidation, warmth, moisture, light, and microorganisms. Ritsert[1a] showed that fat in sealed tubes, both in darkness and in sunlight, remained unchanged, while samples exposed to both air and sunlight absorbed oxygen and became rancid. Schmidt[2] showed that butter made from pasteurized cream and protected from air, light, and warmth remained fresh, while the same butter exposed to air, light, and warmth rapidly became rancid. Von Klecki[3] ascribes to fat-splitting bacteria the principal rôle in the decomposition of butter, and shows that 4 per cent sodium fluoride prevents the formation of acid. Browne[4] states that the factors most active in the decomposition of pure butter fat are air, light, and warmth. Microorganisms are excluded, as the work of Ritsert[1] has shown that bacteria will not grow in pure fat. Laxa,[5] Schreiber,[6] Koenig, and Spiechermann and Bremer[7] have demonstrated the ability of certain microorganisms, especially the molds, to break up fats with the liberation of free acid. While this work has contributed much valuable information on the nature of the changes in butter, the explanations offered for these changes have been insufficient to explain them fully or have not been supported by proper proofs.

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[a] Figures refer to bibliography at end of bulletin.

A recent work by Jensen[8] does much to harmonize the conflicting views of the earlier workers and proves conclusively the causal relation of organisms to the ordinary decomposition. He found that while butter exposed to sunlight was distinctly oxidized, it was only slightly hydrolized. On the other hand, unsterilized butter held in darkness at room temperature was not oxidized, but the acidity was materially increased. In sunlight the bacteria decreased rapidly, while in the portion held in darkness they reached high numbers, but dropped to the extent of 4,000,000 to 7,000,000 per gram at the end of four weeks. In unsterilized butter at room temperature, the acidity increased much more rapidly when it was exposed to the air, beginning first in the outer layer and gradually extending inward.

In the inner part of the butter, the lactic acid bacteria and the yeasts multiplied for a short time, but soon began to decrease; liquefying bacteria and *Oidium lactis* decreased from the beginning. In the surface layer there occurred, in correspondence with the increase of acidity, an increase of short duration of *Bacillus fluorescens liquefaciens*, and often also *B. prodigiosus; Oidium lactis*, *Cladosporium butyri*, and yeasts grew much more slowly, but soon suppressed all others. In butter kept under anaerobic conditions, these forms decreased rapidly, and the flora was soon made up exclusively of lactic acid bacteria and yeasts. The acidity increased only slightly. In a portion protected from the air, the "degree of acidity" increased in ten weeks only from 3.0 to 9.0. By inoculations made in sterile cream butter, Jensen shows that, of the various organisms occurring in butter, only *Bacillus fluorescens liquefaciens*, *Oidium lactis*, and *Cladosporium butyri* were able distinctly to increase the acidity of butter. He thus shows that the action of the air is only indirect in that it does not itself increase the acidity of the butter, but only supplies oxygen to the aerobic organisms which bring about the increase in acidity.

Jensen's results explain very satisfactorily the decomposition of European butter, which is usually put up in small packages with a considerable surface more or less exposed to the air. However, his conclusions can hardly be applied to American butter which is packed, as a large part of it is, in large tubs with a relatively small area exposed to the air. They certainly will not explain the changes in canned butter. This butter is ordinarily put up when perfectly fresh in small cans containing one-half to 3 pounds, completely filled and hermetically sealed. The very small amount of oxygen left would doubtless be quickly used up by the large number of lactic acid bacteria normally present in fresh butter, thus creating a condition under which the aerobic organisms mentioned by Jensen would be unable to live.

The cans usually have the packers' statement that, since they are hermetically sealed, the contents will keep indefinitely, a statement that

would be correct if all oxygen were absolutely excluded and strictly aerobic organisms were the only cause of the decomposition of fat. That this is not entirely true is conclusively proved by the condition of canned butter after keeping it a few months in a warm climate.

THE CONDITION OF OLD CANNED BUTTER.

When this work was started, there were in the Dairy Division of the Bureau of Animal Industry a number of cans of domestic canned butter collected by agents of the division in China, the Philippines, Cuba, and Porto Rico. In most cases the history of the individual cans was not known, further than the place of manufacture and sale, but nearly all had been at the Washington office for about one year. Notwithstanding the fact that the seals were intact, all of these butters showed more or less decomposition. The texture was usually pasty, the aroma somewhat acid and penetrating, the taste, while not what is usually described as rancid, was a disagreeable, biting flavor, corresponding more closely with the so-called "fishy" flavor. The intensity of the "off" flavor varied considerably in the different samples, but none of these butters was fit for use. The acid numbers of a few of these butters are here given:

TABLE I.—*Acid number of old canned butter.*[a]

Sample number.	Acid number.
19	2.6
21	4.7
62	6.1
63	6.0

[a] Method given by Association of Official Agricultural Chemists.

The increase of the acid number above the normal (0.4 to 0.8) was distinct but relatively small when the age of the samples is considered. The iodine number was not determined. Bacteriological examinations were made of a large number of these cans, but, as would be expected, with negative results. Gelatin plates, both aerobic and anaerobic,[a] developed only a very few bacterial colonies, while molds and related groups appeared so infrequently that they could be considered entirely as contaminations. A large percentage of the bacterial colonies were persistent spore-forming species, frequently of the liquefying hay bacillus type. It would, indeed, be surprising if any but the spore-forming varieties were found in a medium containing the amount of free acid usually present in these butters.

[a] Aerobic plates are those so arranged that there is a free access of air to the growing colonies. Anaerobic plates, on the other hand, are held in an atmosphere of an inert gas, such as hydrogen or nitrogen, to allow the development of bacteria which are unable to grow in the presence of free oxygen.

CAUSES OF THE INCREASE IN ACIDITY.

(*a*) PHYSICAL AND CHEMICAL AGENTS.

As has already been noted, many of the earlier writers considered certain physical and chemical agents as important factors in the decomposition of fats. On account of the peculiar conditions under which the butter under consideration is held, only two of these agents—warmth and moisture—can be considered. Light is, of course, excluded, and the effect of oxygen is doubtless reduced to a minimum.

Browne[10] states that the three factors most active in the decomposition of pure butter fat are air, light, and warmth, but that the decomposition may go on slowly if one or even two of these factors are suppressed. Berthelot[11] has shown that fat is broken up when exposed with water to high temperatures, and he thought that this action might go on slowly at ordinary temperatures. That the action is not perceptible at ordinary temperatures is shown by Table II. No. I was a mixture of 20 c. c. of sterile milk and 125 c. c. of butter fat. The action of bacteria was prevented by holding it at 47° C. No. II was a flask of butter partially sterilized by holding thirty minutes in a steam bath and with the action of bacteria prevented by the addition of thymol in the proportion of 1:100.

TABLE II.—*Influence of physical and chemical agents.*

	Acid number.		
	Initial.	29 days.	49 days.
I. Sterile butter fat and milk at 47° C	0.6	0.7	0.9
II. Sterile butter at 23° C	1.1	1.2	1.2

In the earlier part of this work thymol was used because it is usually considered efficient in small quantities, and with most enzymes its inhibitory effect is less than that of formaldehyde. However, on account of its tendency to combine with fat and its slight solubility in water, it is not always efficient in fatty mixtures in proportions greater than 1:200. In this work its antiseptic effect was tested in all cases by bacteriological examinations. Formaldehyde was substituted because, while it has been shown by Kastle and Loevenhart to have little or no effect on lipase, it is an efficient germicide in very small quantities, does not unite with fat, and is readily soluble in water.

In the fat held at the higher temperature there was a questionable small increase of acidity, while at 23° C. the change was within the limits of experimental error. Similar results were obtained from butter made under laboratory conditions from cream which had been held fifteen minutes at 70° C. The usual precautions were taken to

prevent contamination, and the butter sealed in small tubes was held at 23° C. At the end of one hundred days the acid number remained unchanged.

It is therefore evident that the decomposition of butter held under anaerobic conditions is due, not to the action of physical agents, but to some factor which is eliminated or destroyed by a comparatively low temperature. The nonspore-forming organisms and the fat-splitting enzymes are the only known factors which could be included in this class.

The fat-splitting enzyme, or group of enzymes, while it has received comparatively little attention, doubtless plays a very important part in the nutrition of both plants and animals. It is a soluble body of unknown chemical composition, excreted by certain cells or organs, and transforms fats into compounds suitable to be utilized. In the higher plants it transforms the insoluble oily and fatty reserve food into soluble acids and glycerin that may be transported by the sap to the point of growth. An enzyme may act for an indefinite time on a proportionally large amount of material without loss of matter or energy. A small amount of a fat-splitting or lipolytic enzyme if present in butter might, in the course of time, liberate an appreciable amount of acid and destroy the desirable flavor of the butter.

(*b*) THE DIRECT ACTION OF CELLS.

If bacteria or other microorganisms are responsible for the changes in these butters, it is probable that they will be found only while the butter is comparatively fresh. We have seen, at least, that they were not present after the decomposition was well advanced. For the purpose of making a biological study of freshly canned butter one dozen cans were obtained from a factory in Iowa. These were all from one churning of butter, packed while fresh, in the usual way, in 1-pound tin cans, and shipped at once in a refrigerator car. They were seven days old when received, and were probably only slightly changed. The temperature of the laboratory where they were stored was, of course, subject to more or less variation, but most of the time was above 20° C. The condition of the butter is given in the following table:

TABLE III.—*Showing progressive change in canned butter.* (*Series 5.*)

Can No.—	Age in days.	Acid number.	Conditions.
5	7	.....	Flavor and aroma good.
7	18	.....	Not noticeably changed.
8	25	1.0	Slight "off" flavor.
9	32	1.2	Distinct "off" flavor.
10	91	2.1	Distinct fishy flavor.
11	116	2.7	Disagreeable taste; texture pasty.
12	297	3.8	Disagreeable, fishy flavor; penetrating odor.

There was a slow but gradual change in the flavor and general appearance of the butter; an "off" flavor could be detected when it was twenty-five days old, and at the end of thirty-two days it had become quite decided; at one hundred and sixteen days the texture was soft and pasty, the aroma strong, and the taste disagreeable.

It will be noticed that the increase in the acid number corresponded, in a general way, both in this series and in series 22, Table V, with the progressive change in flavor.

Aerobic and anaerobic lactose gelatin plates were made from time to time, a new can being opened for each examination. There was apparently no difference between the aerobic and anaerobic plates except that the numbers were usually smaller on the anaerobic set. Yeasts and similar organisms were determined by adding to gelatin sufficient tartaric acid to inhibit the growth of bacteria without preventing the development of yeast colonies.

TABLE IV.—*Bacteria and yeasts per gram of butter.* (*Series 5.*)

Age in days.	Total.	Lactic.	Liquefiers.	Torula yeasts.
7	5,351,130	5,326,100	.....	24,550
11	3,012,600	2,823,600	6,000	183,000
18	92,700	84,200	.....	8,500
25	12,460	12,000	460	Very few.
91	18,350	17,850	500	00
116	675	00	200	00
[a]297	.....	.....	.....	.....

[a] Sterile.

It is evident that at the time the first examination was made the butter was considerably past its maximum bacterial content, as the total had already dropped to a comparatively low number. As is the case with ordinary butter, the lactic bacteria made up over 99 per cent of the total number and decreased somewhat rapidly until, when examined at one hundred and sixteen days old, they had completely disappeared. Bacteria of the liquefying group were present in small numbers and, while they decreased somewhat, were more persistent than the other forms on account of the high percentage of spore-formers. There were present, in addition to the three groups enumerated in the table, a small and varying number of inert bacteria, which were not accurately counted because of the difficulty in distinguishing their colonies from the other forms, especially from the lactic group. A large number of cultures were made from each class of bacteria, with the hope of finding some form that would account for the change in the butter, but a large part of them proved to be merely slow acid formers, and none were found capable of changing butter fat. On the first set of plates there occurred a few colonies of a yeast-like hyphæ-forming fungus, probably belonging to the Oidium group, but this did not appear again.

The only feature of the flora that could be considered peculiar was the presence in considerable numbers of the Torula[a] yeast group. The negative character of this class of organisms makes it difficult to say definitely if one or several species were present, but in this case the entire group seemed to be made up of one species, distinguished by its small elliptical cells and possessing, in common with other Torulas, the tendency to form, on gelatin, small round white or straw-colored colonies, differing from the lactic colonies only in being slightly larger.

The first set of plates did not contain sufficient acid to inhibit all of the lactic forms completely; consequently the numbers given are not accurate and the apparent increase in the first few days may not be a real one. Like the other nonspore-bearing forms, this group decreased quite rapidly and had almost entirely disappeared in twenty-five days. The species of Torula predominating in this set of butters was recorded in the laboratory as "111 f," but, for convenience, it will be designated as "T" in this report.

For the purpose of confirming these results, and especially to determine if T was a normal inhabitant of canned butter, a second lot of butter was obtained from the same creamery. These cans were packed in September, 1902, from the same churning of fresh butter, and shipped at once in a refrigerator car. They were seven days old when received. For seventy-three days after they arrived they were stored in the laboratory, and at the end of this time were transferred to an incubator held at a constant temperature of 23° C. The following table gives the condition and acid number of each can at the time it was opened:

TABLE V.—*Showing progressive change in canned butter.* (*Series 22.*)

Can No.—	Age in days.	Acid number.	Condition.
22	7	0.4	Flavor clean; texture good.
23	10	0.4	Slight, sharp after-taste.
24	14	0.4	Rather sharp but not distinctly "off."
25	21	0.8	Not distinctly "off."
26	38	1.1	Slight fishy flavor.
27	114	2.0	Disagreeable taste; texture tallowy.
28	251	3.4	Distinct but not strong fishy flavor; sharp odor.

[a] In consideration of the present unsettled position of this group in its relation to other plants, it may be well to state that in this paper the classification of Hansen[12] is followed. He includes in this group those organisms which multiply by budding but do not, like the true yeasts, form spores. The formation of films on the surface of fluid media, while common among the true yeasts, or Saccharomycetes, is infrequent among the Torulas. It seems probable that the Torulas may be only the spore stage, or perhaps degeneration forms, of some of the higher fungi, but for the present it is convenient to classify them with the yeasts.

The butter in this series changed in the same general way as that of series 5, although the change was somewhat slower. In a few days it had the taste and appearance of butter not perfectly fresh, but was without the flavor peculiar to canned butter in the early stages of decomposition. In the can opened when thirty-eight days old, there was the typical "fishy" flavor, and No. 27 had the diagreeable biting taste usually encountered in the old canned butters previously examined. For the first week after removal from cold storage there was no appreciable change in the acidity, and even at the end of two hundred and fifty days the acid number was only seven or eight times that of normal butter. The results of the bacteriological examinations follow, made, as before, at the time each can was opened:

TABLE VI.—*Bacteria and yeasts per gram of butter.* (*Series 22.*)

Age in days.	Total.	Lactic.	Liquefiers.	Torula yeasts.
7	362,000	318,000	21,000	23,000
10	194,100	173,500	3,300	17,300
14	125,000	122,300	2,400	300
21	23,600	23,040	.....	560
114	200	00	150	00
[a]251	.....	.....	.....	.....

[a] Only very few liquefiers.

The results agree quite closely with those of series 5, except that the butter was more advanced when received and the numbers in all the groups were considerably smaller. This was probably caused by a somewhat higher temperature in transit. The Torula group was apparently made up, as before, of the T type.

A third sample of canned butter, while it was not fresh at the time it was examined, was still in the early stages of its decomposition. The original source of the butter was not known, but it had been stored in wood since the summer of 1902 as "Extra June Creamery," and was bought and packed in 3-pound tin cans for the Navy Department in the following February or March. The can examined had been in the Dairy Division at room temperature since repacking.

At the time the butter was packed in tin it was examined by an agent of the Dairy Division and was then in good condition. This can, while not badly "off," had the peculiar fishy flavor of canned butter. It had an acid number of 1.3. Acid gelatin plates developed a few colonies of a Torula yeast, differing morphologically from T but agreeing with it in being able to split butter fat.

A fourth lot of canned butter, consisting of 3-pound tins, from a quantity packed for the Navy Department under the general supervision of the Dairy Division, was received when only a few days old

and held at 23° C. The results of the examination of three of these cans are given in the next table:

TABLE VII.—*Changes in butter of series 66.*

Age in days.	Acid No.	Condition.
7	0.5	Flavor fair.
19	0.5	Not fresh, but not distinctly "off."
29	1.4	Slightly "off" flavor.

This change agrees with that of the two lots given in Tables III and V. At the time the first examination was made the lactic acid bacteria had already dropped to less than 1,000,000 per gram. There were in each of these three cans a few hundred yeasts per gram, divided among four or five species. Of these, two were able to develop acidity in butter fat, but both belonged to the Oidium class and occurred in small numbers only. There were one or two species of Torula, but none of those isolated was able to decompose fat.

It is quite evident that the microscopic life existing in the butter can be considered as only indirectly responsible for the change in the acidity or the flavor. In series 22 there was no perceptible change in the acid number until the bacteria had reached unimportant numbers and the yeasts had nearly disappeared. In series 5, in which the acidity was not determined in the first few cans opened, there was no marked change until both bacteria and yeasts had nearly reached their minimum number. Of the bacteria persisting for any length of time only the lactic group was present in sufficient numbers to be considered as a possible cause. No member of the group has ever been reported as having a fat-splitting ability, and none of those isolated from these samples was able to increase the acidity of butter fat. As will be shown more fully in another section, the Torula T may decompose fat slowly. Assuming that this species is always present in freshly canned butter in considerable numbers, and that it is able to liberate fatty acid by the direct metabolism of its cells, the real change could not be accounted for in this way. Even after the yeasts have entirely disappeared and the bacteria have been reduced until practically nothing remains but resistant spore-forming species, the increase in acidity goes on slowly and steadily, accompanied by a corresponding change in the flavor, aroma, and texture.

This brings us to a consideration of the lipolytic, or fat-splitting, enzymes. It is indeed difficult to understand how fats could be utilized by a vegetable cell without being first broken up into more soluble compounds through the agency of an enzyme.

(c) LIPOLYTIC ENZYMES.

It is probable that there was in these canned butters an enzyme with a weak hydrolyzing action. The presence of an enzyme of this type would offer a very satisfactory explanation of the slow change that goes on after the butter has become practically sterile. If this hypothesis is a correct one, butter that had been heated sufficiently to destroy the enzyme should remain unchanged, while in butter in which the action of microorganisms is eliminated by the addition of a suitable antiseptic the decomposition should go on normally, or at least should be only slightly checked. Table VIII gives the results of a series arranged to determine if lipolytic enzymes were present in canned butter. In making up this set, butter from a can of series 5 was melted at 50° C., and six 50 c. c. Erlenmyer flasks were completely filled. Two of these were sealed without heating or the addition of antiseptic, to two others was added thymol in the proportion of 1:100, and two others were heated in a steam bath thirty minutes and thymol added as in the second pair. Gelatin plates made when the flasks were opened showed that the butter was practically sterile.

In preserving milk or its products with antiseptics it is rarely possible to secure complete sterilization, even in long periods of time, on account of the large number of resistant spore-bearing bacteria normally present in milk. The presence of a few colonies of bacteria of this class on plates made from butter containing an antiseptic indicates that growth was prevented but that the spores originally present were not destroyed.

All of these flasks were held at 23° C., and one flask from each set was examined at twenty-seven and at fifty-four days.

TABLE VIII.—*Test for presence of lipolytic enzyme in canned butter.*

Age in days.	Acid number.		
	Not heated; no antiseptic.	Not heated; antiseptic.	Heated; antiseptic.
Initial.	1.1	1.1	1.1
27	1.4	1.4	1.2
54	1.6	1.6	1.2

An examination of this table shows that the liberation of fatty acids was not checked by the addition of the antiseptic, but, on the other hand, the change was largely inhibited by heating the butter. In other words, the decomposition was brought about, not by the action of chemical or physical agents which would not be affected by the heating, nor by the activity of microorganisms which would be excluded by the addition of antiseptic, but probably by an enzyme unaffected by the antiseptic but destroyed by the heat.

Enzymes of the lipolytic class occur widely distributed in nature, having been found in many organs and secretions of the body, in plants, especially in germinating seeds having a high oil content, and in some of the molds.

An enzyme could be produced in butter in one or both of two ways: (1) It might be produced in the milk or butter itself by microorganisms, or (2) it might be secreted in the udder with the milk and carried over into the butter.

(1) *Enzymes from microorganisms.*—Of the plants known to produce lipase, only the molds and certain bacteria are found in butter. By filtering milk cultures of *Bacillus fluorescens liquefaciens* and *Oidium lactis* through a Chamberland filter and adding the filtrate to butter fat, Jensen[8] obtained a slight increase in the acid number, due, he thinks, to the presence of lipase. These two, as well as the true molds, in which the secretion of lipase is known to be quite common, are aerobes and should not exist in butter after it is canned. None of this class of organisms was found in appreciable numbers in any of the canned butters examined.

Many bacteria isolated from series 5 and 22 were tested in various ways for fat-splitting ability, but with negative results. On the other hand, the predominating yeast, T, was found to have a weak but distinct lipolytic action. This action was determined by adding to 20 c. c. of sterile butter fat 6 to 8 c. c. of a culture grown five or six days in sterile milk at 30° C., mixing thoroughly by shaking in cold water, and incubating at 23° C. The acid number was determined from time to time, using a separate flask for each determination, and making, at the same time, gelatin plates to ascertain the purity of the culture. The rate of increase of free acid under these conditions was found to be as follows:

TABLE IX.—*Increase of acid number by action of T.*

Age in days.	Acid number.
0	0.6
13	3.5
27	4.4
42	9.3

Check flasks, made by adding sterile milk to fat, showed no increase in acidity. In this set action of the living cells is not separated from enzymic action that may be present, but the ability of this Torula to produce a lipolytic enzyme may be demonstrated in a very simple way. A small amount of butter fat was emulsified with melted agar and a loopful transferred to a flamed cover glass. After the agar had solidified it was inoculated by transferring on the point of a platinum needle a very small amount of agar culture, and then the

cover glass sealed with vaseline on a drop-culture slide. In twenty-four hours a small colony had developed about the point of the inoculation. By observing it from day to day with a low magnification, the fat droplets in the vicinity of the colony could be seen gradually disintegrating until, after several days, it was surrounded for some little distance by a clear zone. This is also shown by the effect on butter fat of an old milk culture in which the action of the cells was eliminated by the addition of an antiseptic. A culture was grown in milk one month under favorable temperature conditions, one portion heated ten minutes at 80° C. and formaldehyde added to each part in the proportion of 1:1500. Fat previously heated in a steam bath was added, the mixture sealed in small flasks, and held at 23° C. At the end of seventy-one days, when the acidity was determined, gelatin plates showed that both flasks were sterile. The results follow:

TABLE X.—*Showing presence of enzyme in culture of T.*

Age in days.	Acid number.	
	Not heated; antiseptic.	Heated; antiseptic.
0	0.92	0.92
71	57.56	2.48

There was a slight increase in the acid number of the heated check, caused possibly by the development of acidity in the milk culture before the addition of the antiseptic, or by the splitting of the small amount of fat left by incomplete skimming; or it may have been that the enzyme was not completely destroyed by the ten-minute exposure to 80° C. More recent work, not incorporated in this paper, indicates that the latter is probably the correct explanation. In any case the marked increase in the acid number of the unheated portion could have been brought about only by the elaboration of a lipolytic enzyme by T.

Morphologically, this organism is an elliptical-celled Torula yeast, budding at the ends, with little tendency to form chains or clusters. In young cultures the cells are uniform in form and size, usually varying from 3.6 to 4.5 μ long by 1.8 to 2 μ broad.

Sugars are not fermented. Milk at 30° C. is digested very slowly without previous curdling. In neutral bouillon cultures it is destroyed by an exposure of ten minutes to a temperature of 53° C., or of one minute to 58° C. Colonies do not develop in gelatin containing 1.4 per cent lactic acid nor in gelatin containing 0.2 per cent butyric acid. It grows readily under both aerobic and anaerobic conditions.

The ability to split fat does not appear to be a constant one, but varies with some unknown factor, possibly some slight change in the compo-

sition of the media that would affect the nutrition of the cell. This yeast may multiply in butter for some time without producing any appreciable change in the acidity. Mixtures of butter fat with milk cultures may or may not increase in acidity, and inoculation experiments with sterile cream butter do not always give positive results.

In making inoculated butter the cream was heated sufficiently to destroy the yeasts, but not enough to secure sterilization. For this purpose 4 liters of cream were heated in a large flask ten minutes at 60° C., and, after cooling, inoculated with a pure culture of a lactic acid bacterium. The cream was divided, and to one-half was added a milk culture of T. On the following day it was churned in the flasks, the buttermilk drained off as thoroughly as possible, and the butter washed with boiled water. It was then melted at 40° C., thoroughly mixed, and transferred to small Erlenmyer flasks. It was necessary to melt the butter in order to lessen the chances of contamination in transferring, and to pack it in the flasks without air spaces. It was thoroughly mixed in the small flasks by shaking in cold water. The flasks were held at 23° C., and a separate flask used for each examination.

TABLE XI.—*Yeasts and acidity of experimental butter.*

Age in days.	Inoculated.		Check.	
	Yeasts per gram.	Acid number.	Yeasts per gram.	Acid number.
1 c. c. cream.	302,500	.....	00	.....
2	4,447,000	0.46	00	0.46
7	2,337,000	0.46	00	0.44
13	2,383,000	0.33	00	0.36
32	1,839,000	0.43	00	0.41

The check butter remained free from all organisms that were able to grow on an acid medium and showed no change of acidity. In the inoculated butter the Torula multiplied and maintained a high number for some time, but without causing any appreciable change in the acid number.

A second lot of butter made under very similar conditions gave quite different results.[a] About 3½ liters of fresh cream were heated for 15 minutes at 60° C., divided into equal portions, and to each was added 200 c. c. of a pure culture lactic-acid bacillus grown twenty-four hours in sterile milk. There was also added to one portion 200 c. c. of a culture of T that was grown forty-eight hours in sterile milk. On the following day the two lots were churned by shaking in flasks, and washed and drained with as little exposure as possible. Salt was added in the proportion of 1:48. Both portions were melted in a

[a] Fresh cream for this purpose was supplied by the Dairy Department of the Maryland Agricultural Experiment Station, and the courtesy is hereby acknowledged.

water bath held at 40° to 45° C., and transferred to small flasks, which were sealed with paraffin. The butter was thoroughly mixed by shaking in cold water and was held at 23° C.

TABLE XII.—*Yeasts and acidity of experimental butter.*

Age in days.	Inoculated.		Check.	
	Yeasts per gram.	Acid number.	Yeasts per gram.	Acid number.
Initial.	321,000	0.29	00	0.20
39	Very few.	10.43	00	0.74
42	10,300	9.98	00	0.69
58	28,500	14.13	00	0.78

There was in this case a marked increase in the acidity of the inoculated butter. The Torula T was found to be present in each of the four inoculated flasks, while the check flasks were entirely free from yeasts. The acidity of the check butter increased slightly from the low initial number, but remained stationary at about the normal acid number of fresh butter.

This particular organism which we have found capable of increasing the acid number of butter may be considered as a type of a class that may, under certain conditions, grow in canned or packed butter and, by the secretion of a fat-splitting enzyme, bring about undesirable changes. The yeasts and the related species of the Torula, Oidium, and Monilia type are widely distributed and need only certain favorable conditions for rapid multiplication.

The butter of series 66 probably gives us an example of a decomposition due entirely to the action of organisms of this class. This butter was made from cream heated to 82° to 85° C. in a continuous pasteurizer at skimming stations. This temperature would exclude the possibility of a milk enzyme, and the enzyme which was evidently present must have been produced after the pasteurization by some of the fat-splitting yeasts or molds.

The anaerobic forms, such as the Torula under consideration, would find especially favorable conditions in the highly acid starters commonly used in creameries. The yeasts as a class are favored by an acid medium, and would not, like the great majority of bacteria, be checked by the formation of lactic acid by the sugar-fermenting bacteria. They are even more resistant to lactic acid than the lactic acid bacteria, and would thrive in milk after the latter had been destroyed by the results of their own metabolism. On the other hand, we have seen that this Torula is apparently quite sensitive to some of the fatty acids, such as butyric, and its growth in butter would doubtless soon be checked by the presence of free acids of this class. In view of their weak fat-splitting ability and their presence in canned butter in

comparatively small numbers, this class of organisms can hardly be considered as the only cause of the uniform increase in acidity which has been found to occur in canned butter.

(2) *Enzymes from the milk.*—Mafan and Gilett[13] have demonstrated the presence of a lipolytic enzyme in cow's milk. Spolverini[14] confirmed the results obtained by Mafan and Gilett, and showed that the fat-splitting activity of cow's milk is much weaker than that of human milk and of certain animals.

To test the action of this enzyme on butter fat, fresh milk was obtained from a healthy cow at the Experiment Station of this Bureau. Part of this was heated twenty minutes at 95° to 99° C., and formaldehyde added to each portion int he proportion of 1:1,250. An equal volume of butter fat, which had been previously heated thirty minutes at 95° to 99° C., was mixed with the milk by shaking in cold water and both flasks incubated at 30° C. Gelatin plates made at the end of thirteen and nineteen days showed both flasks to be nearly sterile. The results of the acidity determinations are given in the table following:

TABLE XIII.—*Increase of acidity by milk enzyme.*

Age in days.	Acid number.	
	Heated.	Unheated.
Initial.	0.47	0.47
13	0.45	0.96
19	0.41	1.35

The acid number of the heated check portion remained unchanged, while that of the unheated milk increased distinctly, indicating the activity of an enzyme secreted with the milk.

An enzyme would doubtless be carried over into the butter, and, although present in small amounts, would be able to bring about the slow decomposition already noted as occurring in canned butter.

In order to determine the direct effect of this enzyme on butter a small lot of butter was made in the laboratory under conditions that excluded the possible action of organisms. About 3 liters of cream, separated from morning's milk, were carried at once to the laboratory,[a] divided into equal parts, and the enzyme in one-half destroyed by holding at 60° C. for fifteen minutes. Each portion was cooled to 12° to 13° C. and churned in a large flask, washed in distilled water and thoroughly drained. They were then melted in a water bath held at 45° C. and salted in the proportion of 1:24. Formaldehyde was added to each portion so that the butter would contain approximately 1 part formaldehyde in 1,500 parts of water. This butter was all held in

[a] There was necessarily an interval of several hours between milking and the time of use in laboratory. The possibility of the elaboration of an enzyme by microorganisms was reduced to a minimum by holding the cream at a low temperature.

small sealed flasks at 23° C. The acid number of each portion was determined at the time the butter was made, at the end of forty-eight days, and again at the end of ninety-two days.

TABLE XIV.—*Showing increase in acidity in butter caused by enzyme of milk.*

Age in days.	Acid number.	
	Heated.	Unheated.
0	0.44	0.58
48	0.33	1.76
92	0.48	3.07

The increase in acidity of the unheated portion was small but still sufficient to show a distinct lipolytic action which would in time bring about all the acidity found in ordinary canned butter.

In the experimental butter given in Table XI the milk enzyme was evidently destroyed by the exposure of ten minutes to 60° C. The optimum temperature for lipase is given by Green[14] as 55° C., but this is evidently too high for the lipolytic enzyme of milk. The thermal death point of this enzyme was determined by immersing a small flask containing 15 c. c. of milk in a water bath and holding it at a definite temperature ten minutes. At the end of this time it was cooled at once, formaldehyde added in the proportion of 1:1,200, and the milk mixed with 30 c. c. of butter fat. These flasks were held at 23° C. and the acid number determined at the end of thirty-one days. The results were as follows:

TABLE XV.—*Thermal death point of milk enzyme indicated by acid numbers.*

Age in days.	Unheated.	45° C.	50° C.	55° C.	60° C.	65° C.
Initial.	0.43	0.43	0.43	0.43	0.43	0.43
31	1.17	0.86	0.90	0.75	0.40	0.40

An exposure of ten minutes to 45° C. materially weakened the enzyme, and at 60° C. it was entirely destroyed. It is probable that more careful work along these lines will show distinct differences between lipolytic enzymes from different sources.

In arranging this paper only those results are included which show clearly the points under consideration. In addition to these, there has been obtained a considerable volume of results confirming those incorporated in this paper.

APPLICATION OF THESE RESULTS.

In preparing butter that is to be held for any extended period of time, especially if it is intended for consumption in a warm climate, it is not sufficient that it be merely hermetically sealed. This will

prevent the development of the aerobic forms causing the rapid increase of acidity, but will not exclude certain anaerobic yeasts which may, under certain circumstances, produce a slow development of acidity, nor the action of the fat-splitting enzyme carried into the butter from the milk.

In order to remove these two factors, the milk or cream should be pasteurized at a temperature high enough to destroy the enzyme, and the starter should be prepared and maintained in such a way that the danger of contamination by yeasts will be minimized. The life of unpasteurized butter will be much prolonged by holding it at a low temperature, thus retarding the action of the enzymes.

It is well known among butter dealers that packed butter, when removed from cold storage and held at a higher temperature, commonly develops an undesirable flavor, usually described as "fishy." This is frequently ascribed to the sudden change from a low to a high temperature, but it is much more satisfactorily explained by the activity of the fat-splitting enzymes. This phase of the question will be taken up and will be the subject of a future report.

SUMMARY.

The early investigations were conflicting, but pointed to the importance of light, moisture, heat, oxygen of the air, and microorganisms as factors in causing the undesirable changes in butter.

Recent work by Jensen shows that light, heat, and moisture are unimportant factors, and that air is to be considered only as the source of oxygen for certain aerobic organisms, which are the real cause of the decomposition of butter fat. This explains the changes in ordinary butter, but not those of butter packed in large tubs or in sealed cans.

The examination of old canned butter shows a marked change in the texture and flavor accompanied by a comparatively small increase in the acid number, that is, in the amount of free acid liberated by the breaking up of glycerides. (Table I.)

Only a few microorganisms were found, nearly all belonging to the resistant spore-forming group of bacteria.

The causal relation of physical agents, as heat and moisture, to this change is excluded by the fact that sterile butter held for one hundred days at 23° C. showed no increase in acidity.

Two lots of canned butter, received when about seven days old, were held at room temperature and examined from time to time in regard to their condition, acidity, and bacterial content. The condition changed slowly, showing when about twenty-five days old a distinct "off" flavor, which increased in intensity, until, at two hundred and ninety-seven days in one case and two hundred and fifty-one in

the other, there was a disagreeable "fishy" flavor and a strong penetrating odor. There was a correspondingly slow increase in the acid number. (Tables III and V.)

The flora in each case was made up almost entirely of bacteria of the lactic-acid forming class with a comparatively small number of Torula yeasts and a few liquefying bacteria. Both the lactic group and the yeasts decreased rapidly, until at the end of about one hundred days there were present only a few spore-forming bacteria, mostly of the liquefying group. (Tables IV and VI.)

Since the change in the acidity and flavor went on steadily after the bacteria had practically disappeared, it could not have been brought about by the direct action of the living cells.

This leaves as the most probable cause the action of fat-splitting enzymes.

The presence of an enzyme was shown by the increase of acidity in butter in which the action of organisms was suppressed by an antiseptic, while the heated check portion remained unchanged. (Table VIII.)

An enzyme could be introduced into butter through its elaboration by organisms in the butter itself, or in the milk from which the butter was made, or by its secretion with the milk in the udder.

The predominating species of yeasts in the butter examined was a Torula forming a fat-splitting enzyme. This strongly increased the acidity of butter made under experimental conditions, and probably represents a type of anaerobic organisms that may under certain conditions elaborate sufficient enzyme to produce a slow change. (Table XII.)

The presence of a fat-splitting enzyme in cow's milk has been reported. Its influence on the acidity of butter is shown by the increase in the acid number of an experimental butter which was made from fresh unheated cream, and in which the action of organisms was suppressed by the addition of formaldehyde. A check portion, made under identical conditions, except that the enzyme was destroyed by heat, remained unchanged. (Table XIV.)

It appears from this record of investigation that the only rational conclusion is that the changes which ordinarily occur, or which first occur, in canned butter, destroying its fine, fresh flavor and producing other flavors more or less disagreeable, are due to the liberation of free acid, caused mainly, if not wholly, by the action of an enzyme, which, produced in the milk or secreted with the milk in the udder of the cow, is carried over into the butter; or are, in some cases at least, produced in the butter itself through the activity of certain microorganisms. It seems reasonable to presume that the same agents, the enzymes of the milk acting alone or in conjunction with the yeasts and their resulting enzymes, are responsible for the so-called "fishy" flavor in butter packed in large but unsealed vessels.

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